

# APPLICATION UNDER UNITED STATES PATENT LAWS

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Invention: Corynebacterium Glutamicum Strain with Enhanced Secretion Activity

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## This is a:

- ☐ Provisional Application
- ☒ Regular Utility Application
- ☐ Continuing Application
  - ☒ The contents of the parent are incorporated by reference
- ☐ PCT National Phase Application
- ☐ Design Application
- ☐ Reissue Application
- ☐ Plant Application
- ☐ Substitute Specification  
Sub. Spec Filed \_\_\_\_\_  
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## SPECIFICATION

## CORYNEBACTERIUM GLUTAMICUM STRAIN WITH ENHANCED SECRETION ACTIVITY

The present invention refers to a bacterial strain of *Corynebacterium glutamicum*, which natural genes secD and secF are identified, isolated and sequenced for the first time, to genetical modification(s) of these new genes, concerning gene sequences as well as gene expression and the use of such genetically modified bacterial strain for production of desired substances as well as in a reporter system for protein translocation.

Protein export across the bacterial cytoplasmic membrane mainly follows the ubiquitous general secretory pathway (GSP). The protein translocation is catalyzed by a set of membrane spanning heterotrimers, consisting of the proteins SecY, SecE and SecG in such a way that three SecYE dimers are assembled around a putative pore. In contrast to SecY and SecE which are essential for cell viability, SecG is apparently not required for the formation of the SecYE ring structure (Economou, A. (1999), Trends in Microbiol. 7, 315-319), but stimulates protein secretion by supporting SecA function (Economou, A, Pogliano, J.A., Beckwith, J., Oliver, D.B. and Wickner, W., (1995), Cell 83, 1171-1181). The essential peripheral membrane protein SecA is the mechanical motor of translocation, a dimeric molecule which binds with its carboxy-terminal end to the SecYEG complex (Economou, A. (1999), Trends in Microbiol. 7, 315-319). Preprotein translocation is driven by cyclic insertion and deinsertion of SecA into the membrane under ATP hydrolysis (Lill, R., Dowhan, W. and Wickner, W., (1990), Cell 60, 259-269) and strongly promoted by the proton motive force across the membrane (Shiozuka, K, Mitzushima, S. and Tokuda, H. (1990), J. Biol. Chem. 264, 18843-18847). The auxiliary translocase subunits SecD and SecF enhances protein export in a different manor: (i) they regulate the membrane cycling of SecA (Duong, F and Wickner, W. (1997),

EMBO J. 16, 4871-4879), (ii) they improve the export of proteins with defective signal sequences (Pogliano, J.A. and Beckwith, J., (1994a), EMBO J. 13, 554-561), (iii) they stimulate the proton motive force driven protein translocation (Wickner, W. and Arkowitz, R.A. (1994), EMBO J. 13, 954-953) and (iv) they release mature protein from the membrane (Gardel, C., Johnson, K., Jacq A. and Beckwith, J., (1990), EMBO J. 16, 3209-3216). The chaperone SecB has only been found in gram-negative bacteria so far (Fekkes, P. and Driessen, A., (1999), Microbol. Mol. Biol. Rev. 63, 161-173).

In the gram-positive, non sporulating soil bacterium *Corynebacterium glutamicum*, which is of special interest for the industrial production of fine chemicals (Wohlleben, W., Muth, G. and Kalinowski, J. (1993), Genetic Engineering of Microorganisms, pp. 83-133, edited by A. Pühler, New York, Weinheim), cell wall proteins are the major secretion products (Joliff, G., Mathieu, L., Hahn, V., Bayan, N., Duchiron, F., Renaud, M., Chechter, E. and Leblon, G. (1992), Mol. Microbiol. 6; 2349-2362). Due to this lack of extracellular protease activity and its simultaneous ability to secrete large amounts of proteins, *Corynebacterium glutamicum* is an ideal host for the production of heterologous exoproteins, e. g., as shown for the production of a cellulase from *Cellulomonas fimi* (Paradis, F. W., Warren, R.A.J., Kilburn, D.G. and Miller Jr., R.C., (1987), Gene 61, 199-206), an ovine gamma interferon (Billman-Jacobe, H., Hodgson, A.L.M., Lightowlers, M., Wood, P.R. and Radford, A.J. (1994), Appl Env. Microbiol 60, 1641-1645) and a lipase from *S. hyicus* and a thermonuclease from *S. aureus* (Liebel and Sinskey, US Patent 4,965,197).

Enhancing the secretion of heterologous proteins from *Corynebacterium glutamicum* up to now was only achieved by optimizing the expression of the heterologous protein.

Therefore the heterologous protein was fused with a signal sequence of *C. glutamicum* (Liebel and Sinskey, US Patent 4,965,197; Joliff et al., Patent Nr. FR 2,679,922)

- Several genes of the GSP have been cloned and sequenced in  
5 *Corynebacterium glutamicum*, including secY and secA gene (Kobayashi, M., Fugono, N., Asai, Y., Inui, M., Vertès, A.A., Kurusu, Y. and Yukawa, H. (1994), Gene 139, 99-103; Genetic Analysis and Biomolecular Engineering 15: -13), secG (GenBank D14162), and secE (GenBank AF130462).  
10 However, the genes encoding the auxiliary proteins SecD and SecF have not been identified in *Corynebacterium glutamicum*.

- Object of the present invention was to provide a means for production of high amounts of desired substances which can  
15 be easily isolated from the source of production, as well as a system wherein translocation of the produced proteins can be examined.

- This object is met by a genetically modified bacterial strain *Corynebacterium glutamicum* whereby genetical  
20 modification concerns at least one of its genes secD and secF.

- In all bacterial species analyzed so far sec genes are very conserved in their chromosomal arrangement (Siefert, J.L., Martin, K. A., Abdi, F., Widger, W. R. & Fox, G. E. (1997),  
25 J. Mol. Evol. 45: 467-472), particularly secD and secF genes are directly neighboured, e. g. in *Escherichia coli* (Pogliano, J.A. and Beckwith, J., (1994a), EMBO J. 13, 554-561) or *Mycobacterium tuberculosis* (Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D.,  
30 Gordon, S.V., Eigelmeier, K., Gas, S., Barry, E.E.3<sup>rd</sup>, Tekaiia, F. Badcock, K., Basham, D., Brown, D., et al. (1998), Nature 6685, 537-544).

- Corynebacterium glutamicum* secD has a size of 1911 bp. Homology data and possible Shine-Dalgarno sequence AAGGA suggest that the gene starts with the rare start codon TTG. The gene specifies a protein of 637 amino acids with a
- 5 calculated molecular mass of 67.689 and a theoretical pI of 4,52. The protein possesses six putative transmembrane spanning regions in an unregular distribution with an extracytoplasmatic loop of 371 residues, predicted by HMMTOP analysis (Tusnády, G.E. and Simon, I., (1998), J.
- 10 Mol. Biol. 283, 489-506) and six conserved motifs D1-D6, which are present in all known SecD proteins (Bolhuis, A., Broekhuizen, C.P., Sorokin, A., van Roosmalen, M.L., Venema, G., Bron, S., Quax, W.J. and van Dijl, J.M. (1999), J. Biol. Chem. 273, 21217-21224). An alignment of the
- 15 deduced amino sequences of *Corynebacterium glutamicum* and *M. tuberculosis* SecD (Fig. 2a) revealed 41 % identity and an overall similarity of 61 % (Myers, G. and Miller, W., (1988) CABIOS 4, 11-17), but the proteins are not homologous over their entire length. Only the C-terminal
- 20 part with 5 transmembrane regions and the 6 short patterns D1-D6 of corynebacterial SecD are highly conserved, the extracytoplasmatic loop and the elongation of the C-terminus of the protein reveals much lesser conservation to the mycobacterial SecD protein.
- 25 *Corynebacterium glutamicum* secF consists of 1209 bp, starts five bases after the secD stop codon and its putative Shine-Dalgarno sequence AGGAG is part of secD 3'end. The distance between the secD stop codon TAG and the secF start
- 30 ATG is two nucleotides. With 403 amino acid residues the protein shows a calculated molecular mass of 43.664 and a theoretical pI of 5,07. Its structure resembles the SecD protein. It also has six transmembrane spanning regions and an extracytoplasmatic loop of 95 residues but its N-
- 35 terminus is shorter as compared to the mycobacterial SecF (Fig. 2b). Aligned with *M. tuberculosis* SecF, it exhibits 43 % identity and an overall similarity of 60 % (Myers, G.

and Miller, W., (1988) CABIOS 4, 11-17), but as in the case of SecD, the protein is also not conserved over its entire sequence. Like SecD, only the section containing the transmembrane domains and four regions F1-F4, which are  
5 represented in all SecF proteins (Bolhuis, A., Broekhuizen, C.P., Sorokin, A., van Roosmalen, M.L., Venema, G., Bron, S., Quax, W.J. and van Dijl, J.M. (1999), J. Biol. Chem. 273, 21217-21224), are well conserved. Analysis of the SecF amino acid sequence with FingerPRINTscan (Attwood, T.K.,  
10 Flower, D.R., Lewis, A.P., Mabey, J.E., Morgan, S.R., Scordis, P., Selley, J. and Wright, W. (1999), Nucleic Acid Res. 27, 220-225) revealed two highly conserved possible SecY interaction sites (Fig. 2b), which are part of the transmembrane domains I and VI.

15 Protein sequence of SecD is shown as SEQ ID NO. 3, encoded by the polynucleotide sequence SEQ ID NO. 1, protein sequence of SeqF is shown as SEQ ID NO. 4, encoded by the polynucleotide sequence SEQ ID NO. 2.

"Substance" in content of the present invention can be any  
20 product of a pathway of the bacterium, catabolic as well as metabolic pathway, preferably the "substance" is selected from the group amino acid, oligopeptide, polypeptide and protein. Further the substance can be the product of an introduced heterologous gene or can be produced by this  
25 heterologous gene product.

"Homologous protein" or "homologous amino acid sequence" in content with one of the proteins of the present invention means in the present application an amino acid sequence, wherein at least 70 %, preferably 80 %, more preferably 90  
30 % of the amino acids are identical to one of the proteins of the present invention and wherein the replaced amino acids preferably are replaced by homologous amino acids. As "homologous" amino acids are designated which have similar features concerning hydrophobicity, charge, steric features  
35 etc.

"Functional mutants" in the sense of the present invention are proteins with an amino acid sequence according to SEQ ID NO. 3 or 4, whereby a single or several amino acids are artificially or naturally replaced by amino acids with different properties, however, the protein as a whole shows similar characteristics concerning e.g. in domains of the three dimensional structure, functional behaviour or effectiveness in one of the embodiments described in the present application.

10 "Fragments" in the meaning of the present description are polypeptides comprising parts of the amino acid sequence according to SEQ ID NO. 3 or 4 or of a homologous protein or a functional mutant of one of these proteins. Preferably the fragments are functional fragments, which means that they are effectively involved in secreting or in a reporter system described in the present application. Fragments of the protein as well as the whole sequence can further be part of fusion proteins that contain other protein sequences, which foster the level or location of expression or targeting.

To obtain a bacterial strain of *Corynebacterium glutamicum* wherein protein production and/or secretion is enhanced, the genes supporting the production and secretion may be modulated, resulting in a genetically modified bacterial strain according to the invention. "Modification" comprises as well mutation, deletion and insertion of polynucleotides in the genes, as rearrangement of the genes to each other or to their promoters, selection of a suitable promoter, modulating the expression of the proteins, preferably enhancing the expression of secretory proteins, multiplying genes and much further. The modification(s) of the genes supporting the production and secretion of proteins may be located in genes lying in cis or in trans position to each other, modifications may be integrated in the chromosome or may remain on a plasmid.

In one preferred embodiment of the invention at least one of the proteins of the general secretory pathway is overexpressed compared to wild type expression, resulting in an enhanced protein secretion of the bacterium.

- 5 Preferably, at least one of the proteins SecD and SecF are overexpressed, more preferably these two are overexpressed simultaneously.

- Overexpression of the proteins may be obtained for example by setting at least one of the genes secD an/or secF under  
10 control of a strong promoter, preferably by inserting the genes in an expression vector which is transferred into the cell or by multiplication of the genes.

- Preferably, overexpression of at least one of the proteins SecD and SecF is combined with overexpression of at least  
15 one of the essential Sec proteins (SecE, SecY, SecA).

- "Overexpression" of a protein means that this protein is expressed to a higher amount than it is naturally expressed from a wild type *Corynebacterium glutamicum*. Preferably overexpression is at least 1,5 fold the amount compared to  
20 wild type expression, more preferably at least twofold.

The constructed plasmids for overexpression, containing several combinations of sec genes are shown in Table 1, plasmid maps are shown as Figures 5 to 8.

Table 1

- 25 *Corynebacterium glutamicum* strains and plasmids

strains/ plasmids	relevant genotype	reference
strains:		
C. g. RES167	Restriction-deficient mutant of ATCC 13032, $\Delta(cglRI-cglRII)$	Universität Bielefeld



	RES167 <i>secD</i> :: pCR2.1, Km <sup>R</sup>	
C .g. INT-D	RES167 <i>secF</i> :: pCR2.1, Km <sup>R</sup>	This study
	RES167 <i>secG</i> :: <i>cmx</i> . Cm <sup>R</sup>	This study
C. g. INT-F	RES167 <i>dciaE</i> :: pIAmy2, Tc <sup>R</sup> , test strain for amylase excretion	This study
C. g. INT-G		This study
C. g. AMY2	Vector for cloning of PCR products Ap <sup>R</sup> , Km <sup>R</sup>	
plasmids:	pK18mob harbouring <i>dciaE</i> fragment	Invitrogen
	pCR2.1 harbouring <i>secD</i> fragment	Wehmeier et al., (1998)
pCR2.1	pCR2.1 harbouring <i>secF</i> fragment	
pLW60	Mobilizable cloning vector, <i>sacB</i> -derivative, Km <sup>R</sup>	This study
pInsD		This study
pInsF	pSVB31 containing <i>cmx</i> of pTP10, Cm <sup>R</sup>	Schäfer et al. (1994)
pK18mobsac B	pK18mob- <i>sacB</i> harbouring <i>secG</i> :: <i>cmx</i> <i>E. coli</i> expression vector, P <sub>trc</sub> , Tc <sup>R</sup>	Universität Bielefeld
pEC31	<i>E. coli</i> / <i>C. glutamicum</i> shuttle expression vector, P <sub>trc</sub> , Km <sup>R</sup>	This study
pInsG		Universität Bielefeld
pXT99A	<i>E. coli</i> / <i>C. glutamicum</i> shuttle expression vector, P <sub>trc</sub> , Tc <sup>R</sup>	
pEC-XK99A	pEC-XT99A harbouring <i>secD</i> downstream P <sub>trc</sub>	Universität Bielefeld
		Universität Bielefeld
pEC-XT99A	pEC-XT99A harbouring <i>secG</i> downstream P <sub>trc</sub>	
		This study
pSecD	pEC-XK99A harbouring <i>secD secF</i>	

pSecG	downstream P <sub>trc</sub>	This study
pSecDF	pEC-XK99A harbouring <i>secE</i> , <i>secD</i> <i>secF</i> downstream P <sub>trc</sub>	This study
pSecEDF		This study
pSecYDF	pEC-XK99A harbouring <i>secY</i> , <i>secD</i> <i>secF</i> downstream P <sub>trc</sub>	This study
pULMJ95	<i>E. coli</i> / <i>C. glutamicum</i> shuttle vector, <i>amy</i> , Km <sup>R</sup>	Cadenas et al. (1996)
pIAmy2	pEC-XT99A harbouring <i>amy</i> downstream P <sub>trc</sub>	This study
	pXT99A harbouring <i>amy</i> <i>dcIAE</i> -fragment downstream P <sub>trc</sub>	This study

One preferred embodiment of the present invention is a *Corynebacterium glutamicum* bacterial strain transformed by plasmid pSecD.

- 5 Another preferred embodiment of the present invention is a *Corynebacterium glutamicum* bacterial strain transformed by plasmid pSecDF.

Particularly preferred embodiments of the present invention are *Corynebacterium glutamicum* bacterial strains  
 10 transformed by plasmid pSecEDF or pSecYDF, respectively.

A bacterial strain according to the invention may contain besides the genetical modification of one of the described *sec* genes further modifications resulting in enhanced protein secretion. Particularly, a bacterial strain  
 15 according to the invention contains at least one further heterologous gene, preferably encoding a protein which shall be produced in high amounts. Therefore, the heterologous gene is introduced in a bacterial strain according to the invention - after genetical modification

of the sec genes - or an already constructed bacterial strain containing a desired gene is genetically modified concerning the sec genes as described herein.

5 Introduction of the plasmids used in genetical modification steps may be carried out by any method known in the art, for example by transfection, injection, ballistic missile, viral vectors, electroporation,  $\text{CaCl}_2$  method or heat shock.

10 A bacterial strain of the present invention is particularly suitable for amino acid, peptide or protein production, since the produced substances may be secreted from the bacteria in a high amount, and that is why the substances can be isolated directly from medium (supernatant) without cell damage.

15 A bacterial strain of the present invention may further be used in a reporter system. The reporter system may report about gene regulation, protein expression, protein translocation, or inducibility of gene expression. Preferably in the reporter system the produced proteins are translocated over the cell wall, whereby they are easily to  
20 determine in the supernatant.

In one embodiment of such a reporter system protein expression of proteins naturally occurring in *Corynebacterium glutamicum* can be determined by protein characterisation of the secreted proteins.

25 In another embodiment of a reporter system the bacterial strain of the present invention is transformed by introducing a heterologous marker gene into the cell, encoding a marker protein.

30 For examining gene regulation the marker gene is located "behind" an interesting promoter, controlling naturally the examined gene. By this construction it is possible to determine how and under which conditions the examined gene

is expressed, resulting in conclusions about regulation of this gene.

A similar construction involves further regulating elements of a gene, resulting in a system for determining the  
5 inducibility of gene expression of an interesting gene.

In all of the embodiments protein characterization may be carried out by any method known in the art, for example by measuring enzyme activity, SDS-PAGE, sequencing, immunologic methods, i. e. Western blotting, or  
10 chromatographic methods.

Protein production by use of a bacterial strain of the present invention can particularly be enhanced by introducing (a) heterologous gene(s) into the bacterial cell enabling the cell to grow faster. Preferably, such a  
15 heterologous gene enables the cell to use external energy, normally not used by this bacterial strain. Such external energy comprises several sugars, amino acids, peptides, carbohydrates, fatty acids, organic polymers, inorganic ions and light.

20 One preferred external energy source is starch. The bacterial strain of the present invention can be transformed in a way that it is able to use starch as a sole energy source, for example by introducing a heterologous amylase gene.

25 A particularly preferred bacterial strain of the present invention is a *Corynebacterium glutamicum* RES167 strain, transformed by plasmids pSecYDF and pIAmy2.

#### Brief Description of the Figures

##### Figure 1

30 Schematic presentation of the chromosomal organization of the *M. tuberculosis* secD/secF region (a), compared with the

same region in *Corynebacterium glutamicum* (b). In the scheme of the rescued vector (b), restriction enzymes used for plasmid rescue are in brackets and enzymes used for cloning are marked (\*).

## 5 Figure 2

Comparison of the deduced amino acid sequences of SecD (a) and SecF (b) of *Corynebacterium glutamicum* and *M. tuberculosis*. Identical amino acids (\*) and conservative replacements (:) are marked. The conserved regions D1-D6 and F1-F4, which are present in all known SecD and SecF Proteins are boxed. Two possible SecY interaction sites of SecF were found by analysis of the amino acid sequence with FingerPRINTscan (Attwood et al., 1999, above) and marked with black bars. The six putative transmembrane regions of SecD and SecF are indicated in grey shadings. The membrane spanning domains of all Proteins were predicted using the HMMTOP web site (Tusnády and Simon, 1998, above).

## Figure 3

Amylase secretion of *Corynebacterium glutamicum* AMY2 overexpressing different combinations of sec genes. 1,3 X 10<sup>6</sup> cells were incubated for 16 h. Activity in the supernatant was determined 5 times. 1 mU was defined as 1 nmol reducing sugar min<sup>-1</sup> ml<sup>-1</sup>. A significant increase in amylase secretion could be detected if secD and secF are overexpressed (pSecEDF). In *Corynebacterium glutamicum* AMY2/pSecEDF(pSecEDF) and *Corynebacterium glutamicum* AMY2/pSecYDF(pSecYDF) amylase activity is more than doubled compared with *Corynebacterium glutamicum* AMY2(AMY2).

## Figure 4

Growth of different *Corynebacterium glutamicum* strains *Corynebacterium glutamicum* AMY2 (■), *Corynebacterium glutamicum* AMY2/pSecEDF(Δ) and *Corynebacterium glutamicum* AMY2/pSecYDF(●) in minimal medium with starch as sole

carbon source. No growth was detectable for *Corynebacterium glutamicum* RES167 (◆). The slight decrease of optical density at the beginning of the curve results from degradation of insoluble parts of starch by the secreted  
5 amylase.

Figure 5 is a plasmid map of pSecD

Figure 6 is a plasmid map of pSecDF

Figure 7 is a plasmid map of pSecEDF

Figure 8 is a plasmid map of pSecYDF

10 Figure 9 is a plasmid map of pAmy

Figure 10 is a plasmid map of pIAmy2

The following examples shall be considered as explaining the present invention in detail without restricting the scope of the invention.

- 15 All bacterial strains and plasmids relevant for this study are listed in Table 1. *E. coli* and *Corynebacterium glutamicum* strains were routinely cultivated in Luria-Bertani (LB) medium (Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989), Molecular Cloning: a Laboratory  
20 Manual, 2<sup>nd</sup> edn. Cold Spring Harbour, NY: Cold Spring Harbour Laboratory) at 37°C and 30°C respectively. For growth of *Corynebacterium glutamicum* on starch as sole carbon source, a modified minimal medium (Katsumata, R, Ozaki, A., Oka, T. and Puruya, A. (1984), J. Bact. 159,  
25 306-311), containing 2 % soluble starch (Sigma) instead of glucose and yeast extract, was used. Antibiotics used for plasmid selection were kanamycin (50 µg ml<sup>-1</sup>) and chloramphenicol (10 µg ml<sup>-1</sup>).

#### Example 1

## Construction of expression vectors pXT99A and pEC-XK99A

*E. coli* expression vector pTRC99A (Amann et al., 1988, Gene 69: 301-315) was cleaved with BspHI and treated with Klenow fragment. Tetracycline gene from *C. glutamicum* plasmid pAG1  
5 (GeneBank Acc.No. AF121000) was inserted instead of ampicilline' gene by ligation with T4 ligase, resulting in pXT99A. Ligation mix was electroporated into *E. coli* DH5 $\alpha$ MCR.

For construction of pEC-XK99A *E. coli* - *C. glutamicum*  
10 shuttle vector pTRC99A was cleaved with BspHI and treated with Klenow fragment . Ampicilline gene was replaced by kanamycine resistance gene of *E. coli* plasmid pBSL15 (Alexeyev, M., 1995, Biotechniques 18: 52-56). Ligation and electroporation was carried out as described above. Thus  
15 plasmid pXK99A was obtained.

From plasmid pGA1 (Sonnen et al., 1991, Gene 107; 69-74) a 3484 bp fragment containing a replicon of *C. glutamicum* was obtained by restriction with BalI and PstI. This fragment was inserted into SmaI/PstI cleaved vector pK18mob2 (Tauch  
20 et al., 1998, Archives of microbiology 169: 303-312). After religation a 839 bp fragment of the inserted replicon fragment was deleted by cleavage with BamHI/XhoI and the vector fragment was treated with Klenow fragment. After religation of the vector a 2645 bp KpnI/PstI, Klenow  
25 treated fragment, containing *C. glutamicum* minimal replicon was inserted into plasmid pXK99A, cleaved with NheI and treated with Klenow-polymerase. Ligation was carried out as described above. After electroporation into *C. glutamicum* plasmid pEC-XK99A was isolated and verified.

30 Example 2

Isolation and characterization of corynebacterial sec genes:

- E. coli* DH5 $\alpha$ MCR (Grant, S.G.N., Jessee, J., Bloom, F. R. and Hanahan, D. (1990), Proc. Natl. Acad. Sci. USA 87, 4645-4649) was used as host for plasmid construction. Plasmid DNA from *E. coli* was prepared by an alkaline lysis method (Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989), Molecular Cloning: a Laboratory Manual. 2<sup>nd</sup> edn. Cold Spring Harbour, NY: Cold Spring Harbour Laboratory) modified for *Corynebacterium glutamicum* by using 20 mg of Lysozyme ml<sup>-1</sup> of lysis buffer HB1 at 37°C for 2 h.
- 10 Chromosomal DNA of *Corynebacterium glutamicum* was isolated as described by Tauch, A., Kirchner, O., Wehmeier, L., Kalinowski, J. and Pühler, A (1994), FEMS Microbiol. Lett. 123, 343-347. DNA restriction, agarose gel electrophoresis, Klenow treatment and ligation were carried out according to
- 15 standard procedures (Sambrook et al., 1989, above). Enzymes for DNA manipulation were obtained from Pharmacia or Boehringer and used as recommended by the manufacturer. Isolation of DNA restriction fragments from agarose gels was performed by means of the Nucleotrap Extraction Kit for
- 20 Nucleic Acids (Macherey-Nagel).

- All primer sets used for PCR experiments are listed in Table 2. To isolate a promoterless *secY*, the gene was generated by PCR with synthetic oligonucleotides *sy1* and *sy2* (Table 2), deduced from the GenBank entree D14162. The
- 25 small genes *secE* (GenBank AF130462) and *secG* (231 bps, GenBank AJ007732) were directly amplified from the chromosome using the primers *se1* and *se2* to receive *secE* and *sg1* and *sg2* to get *secG*. All PCR generated genes were first cloned into pCR2.1 using the TA Cloning Kit
- 30 (Invitrogen), *EcoRI* digested and cloned into the IPTG inducible *E. coli* / *Corynebacterium glutamicum* shuttle expression vector pEC-XT99A under control of the *trc* promoter in a second step, resulting in the plasmids, pSecY, pSecE and pSecG.



A promoterless *secD* was amplified by PCR using the deduced primers *sd1* and *sd2*, derived from the sequence of the plasmid rescue and cloned as described above.

- PCR was carried out with a PCT-100 Thermocycler (MJ Research, Inc.) with a Taq DNA polymerase (Gibco-BRL). The
- 5 initial denaturation was conducted at 94°C for 2 min followed by 90 s of denaturation, 90 s of annealing at the primer dependent temperature  $T_m$  (2AT+4GC) of -5°C (Suggs, S.V., Hirose, T., Miyake, T., Kawahima, E.H., Johnson,
- 10 M.L., Itakura, K. and Wallace, R.B. (1981), Developmental biology using purified genes. Academic Press, Inc., New York, N.Y., pp. 683-693), and 90 s of extension at 72°C. This cycle was repeated 32 times and completed by an extension step for 10 min at 72°C.
- 15 Plasmids were introduced in *E. coli* and *Corynebacterium glutamicum* by electroporation (Tauch et al., 1994, FEMS Microbiol. Lett. 123, 343-347; Haynes and Britz, 1989, FEMS Microbiol. Lett. 61 329-334)

- DNA sequencing was done by the Institut für
- 20 Innovationstransfer GmbH (Bielefeld). Searches for amino acid similarities were carried out with the BLAST service (Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990), J. Mol. Biol. 125, 403-410), protein alignments were computed by the CLUSTAL W program
- 25 (Thompson, J.D., Higgins, D.G. and Gibson, T.J., (1994), Nucleic Acid Res. 22, 4673-4680).

- In the *Mycobacterium tuberculosis* H37Rv strain, that is taxonomically closely related to *Corynebacterium glutamicum*, the dipeptid transporter encoding gene *dciaE*
- 30 (Fig. 1a) is located downstream of *secD* and *secF*. The *dciaE* homolog of *Corynebacterium glutamicum* (ATCC 13032) was sequenced in part in a study on the *rel* gene (Wehmeier, L. Schäfer, A., Burkowski, A., Krämer, R., Mechold, U., Malke, H., Pühler, A. und Kalinowski, J. (1998), Microbiology 144,

1853-1862) to isolate the genes *secD* and *secF* by a chromosomal rescue technique, an 0,8 kb fragment of the *dcIAE* gene, derived from plasmid pLW60 (Wehmeier et al., 1998) by digestion with *EcoRI* and *BamHI* was cloned into pCR2.1 and the resulting plasmid was integrated into the *Corynebacterium glutamicum* chromosome after electoporation via homologous recombination. Total chromosomal DNA was isolated from the resulting strain, digested with *EcoRV* and *SspI* religated and transferred to *E.coli* DH5 $\alpha$  MCR. The rescue of the integrated vector with *EcoRV* and *SspI* results in a plasmid pCR2.1 carrying a 9751 bp insert including *dcIAE* and the upstream chromosomal region (Fig. 1). The insert was sequenced by primer walking. By DNA sequence analysis, 8 complete and 1 partial orfs ("open reading frames"), including *secD* and *secF* could be identified on the fragment.

Sequences of *secD* and *secF* are shown as SEQ ID NO. 1 and SEQ ID NO. 2, respectively.

Table 2 PCR primers for amplifying corynebacterial *sec* genes

Gene	5'-primer	3'-primer
<i>secA</i> fragment	saf1: 5'-CGCGACAAGGACTACATCGT-3'	saf2: 5'-GAGATGTCTGCGGATTCGAG-3'
<i>secY</i> fragment	syf1: 5'-TGAGGAGGCCAGGAGGCCAG-3'	syf2: 5'-AACCACCGTACTGACGACGA-3'
<i>secY</i>	syl: 5'-TTAAGTGCTGAGGAGGCCAG-3'	sy2: 5'-TTATCAGCACCGGTAGTTCC-3'
<i>secE</i>	se1: 5'-TGGATGAGTAGTGATTTAGA-3'	se2: 5'-GATTCTGACTCCGTAGGTAG-3'
<i>secE</i> region	ser1: 5'-CACCTGGCAGACGCACTCAA-3'	ser2: 5'-AGCCGGAGTAGCACTGAATG-3'
<i>secG</i>	sg1: 5'-ACCTGGGTTCTCAAACGGCA-3'	sg2: 5'-TTGTCGACCTGTTGTCTCCC-3'
<i>secG</i> region	sgr1: 5'-TCCAGGCCTTCTCAGCAA-3'	sgr2: 5'-AGCTGCGAGAATCCAGGCTA-3'
<i>secD</i>	sd1: 5'-TTGTCTGGTTGATTGGAATT-3'	sd2: 5'-TGAAGTTTCAGTCTGGGAAT-3'
<i>secD</i> fragment	sdf1: 5'-TGCTGTGACAGGCGATCGT-3'	sdf2: 5'-TCATCAGTGGTGCATGCAT-3'
<i>secF</i> fragment	sff1: 5'-GTACCAAGATGAGCATGCCA-3'	sff2: 5'-ATCGAACGCATGAAGGTCTG-3'

### Example 3

Construction of plasmids pSecDF, pSecEDF and pSecYDF

The plasmid pCR2.1, carrying chromosomal secD and secF genes on a 9751 bp insert (example 1) was cleaved with SalI and a 3275 bp fragment was isolated by gel electrophoresis. This fragment was inserted into pEC-  
5 XK99A, cleaved by SalI, resulting in plasmid pSecDF. Ligation and electroporation in *E. coli* DH5 $\alpha$ MCR was carried out as described above, bacteria are incubated on LB agar plates containing 50  $\mu$ g/ml kanamycine.

For construction of pSecEDF and pSecYDF, respectively,  
10 pSecDF was cleaved with EcoRI. pSecE and pSecY (example 2), respectively, were also cleaved with EcoRI, whereby the secE and secY gene containing fragments can be isolated. The EcoRI-fragments containing secE and secY genes are inserted into EcoRI-cleaved pSecDF, resulting after  
15 ligation in pSecEDF and pSecYDF, respectively.

#### Example 4

Directed mutagenesis of sec genes in the chromosome of *Corynebacterium glutamicum* RES167

A defined secD mutant was constructed by gene disruption  
20 via homologous recombination. Therefore, a 609 bp internal fragment of secD (nt 1200-1809) was amplified by PCR using the deduced primers sdf1 and sdf2 (Table 2) and cloned into pCR2.1. The resulting plasmid pInsD was electroporated into *Corynebacterium glutamicum* and could only establish itself  
25 by homologous recombination into the chromosome. The gene disruption was verified by Southern hybridisation. A secF mutant strain *Corynebacterium glutamicum* INT-F was built in the same way, using the primers sff1 and sff2 to reveal an internal fragment of 603 bp (nt 346-949), leading to the  
30 plasmid pInsF. For secA and secY mutation, internal fragments of the genes, derived by PCR using the primers saf1, saf2, syf1 and syf2 respectively, were cloned into pCR2.1 but failed to integrate into the chromosome.

Due to the small size of *secG* and *secE* a *secG* mutant was constructed by insertional inactivation using the *sacB* system, which enables the positive selection of allelic exchanges by homologous recombination (Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G. and Pühler, A. (1994), Gene 145, 69-73). For this purpose, a 1,3 kb DNA fragment with a central *secG* was created by PCR with the synthetic oligonucleotides *sgr1* and *sgr2* and subsequently cloned via pCR2.1 using *SalI* and *XbaI* into the plasmid pXT99A. To isolate the chloramphenicol resistance gene cassette *cmx*, vector pEC31 was digested with *SalI* and *HindIII* and the 2,0 kb fragment bearing *cmx* was isolated from an 0,8 % agarose gel. Integration of *cmx* into *secG* was achieved by ligation of the *BspHI* digested plasmid and the *cmx* cassette after Klenow treatment of both fragments. The constructed plasmid was reisolated from *E. coli* and the 3,3 kb fragment containing *secG::cmx* was cloned with *SalI* and *HindIII* into pK18mobsacB (Schäfer et al., 1994). This vector pInsG was integrated into the chromosome of *Corynebacterium glutamicum* in such a way, that the resulting strain carried the modified *secG* region and the wild type gene separated by vector sequence. Excision of the plasmid can be selected for by growing the cells on LB agar containing 10 % sucrose (Schäfer et al., 1994). Cells able to grow on this medium have lost the plasmid due to a second cross-over event that either restores wild type gene arrangements or leads to a selectable chloramphenicol resistant strain *Corynebacterium glutamicum* INT-G, carrying only the disrupted allele of *secG*. The *secG* disruption was verified by Southern hybridisation.

For disruption of *secE* a 1,6 kb DNA fragment was amplified by PCR using the primer *ser1* and *ser2*, derived from the *secE* flanking regions (Wehmeier, 1999) and cloned via pCR2.1 into pK18mobsacB using the enzymes *XbaI* and *HindIII*. The resulting vector was cleaved at the single *BssHII* site within the *secE* gene. Klenow treated, ligated with the *cmx*

gene fragment from pEC31 an integrated into the chromosome of *Corynebacterium glutamicum*. Further steps were carried out as described above, but no double cross-over event could be detected.

- 5 The developed mutant strains *Corynebacterium glutamicum* INT-D and *Corynebacterium glutamicum* INT-F (Table 1) are enlarged in size, showed a significantly prolonged lag phase and did not reach the optical density of the wild type in liquid media. The mutant phenotypes can be  
10 complemented by plasmid encoded, intact sec genes (not shown).

#### Example 5

Construction of an amylase secreting reporter system

- Construction of amylase producing *Corynebacterium*  
15 *glutamicum* strain, the vector pULMI95 (Cadenas, R.F., Fernandez-Gonzales, C., Martin, J.F. and Gil, J.A. (1996), FEMS Microbiol. Lett. 137, 63-68) was digested with *EcoRI* and *Ecl136II* and a 2,1 kb fragment harboring the *amy* gene of *Streptomyces griseus* IMRU 3570 was cloned into the *EcoRI* and *Ecl136II* cleaved *E. coli* / *Corynebacterium glutamicum*  
20 shuttle expression vector pEC-XT99A under control of the IPTG-inducible *trc* promoter (Amman, E., Ochs, B. and Abel, K.-J. (1988), Gene 69: 301-315). The new constructed vector pAmy with IPTG inducible amylase expression was  
25 electroporated to *Corynebacterium glutamicum*.

- For *Corynebacterium glutamicum* strains harbouring a chromosomal copy of the amylase gene, *amy* was cloned as described above into the *E. coli* expression vector pXT99A. In a second step the *XbaI* and *HindIII* gene fragment of  
30 *dcIAE* from pLW60 (Wehmeier, et al., 1998) was cloned into the resulting vectors downstream of *amy*. The new non-replicative plasmid pIAmy2 was integrated into the *Corynebacterium glutamicum* chromosome by electroporation

and following homologous recombination resulting in the strain *Corynebacterium glutamicum* AMY2 (Table 1).

#### Example 6

##### Amylase activity assays

- 5 For performing amylase assay *Corynebacterium glutamicum* strains were cultivated in solid and liquid cultures of TYP5 medium, consisting of 1 % yeast extract (Difco), 1 % peptone (Difco) and 2 % soluble starch (Sigma). Amylase production was induced by adding 50 nM IPTG to the TYP5
- 10 medium. For measurement of intracellular amylase activity, *Corynebacterium glutamicum* grown in liquid culture were washed twice in 10 mM phosphate buffer (pH 7,0). To disrupt the cells a Ribolyser (Hybaid) was used two times for 30 s at a speed of 6.
- 15 Amylase activity was measured by a modification of the dinitrosalicylic acid method (Miller, G. L., 1959, Anal. Chem. 31, 426-428) in the supernatant of *Corynebacterium glutamicum* strains. The assay was carried out at 37°C for 30 min with 2 % soluble starch in 10 mM phosphate buffer
- 20 (pH 7,0). The volume activity (mU) was defined as nmol reducing sugar min<sup>-1</sup> ml<sup>-1</sup>. Starch degradation was assayed on agar plates by colouring with Lugols solution. Amylase activity was detected as clearing zones around the colonies.
- 25 Starch degradation on agar plates was detectable for *Corynebacterium glutamicum* AMY2 and amylase activity in the culture supernatant after three days of incubation was 45 mU. *Corynebacterium glutamicum* AMY2, bearing a chromosomal copy of amy, secretes only 12 % of the amylase produced by
- 30 *Corynebacterium glutamicum* RES1167/pAmy. This led to the conclusion, that the plasmid pAmy has around 8 copies per cell in *Corynebacterium glutamicum*. In opposite to the wild type strain, all amylase producing *Corynebacterium*

- glutamicum* strains are able to grow on minimal media with starch as only carbon source. It was concluded that amylase production is easy to assay in both, a replicative and an integrated system in *Corynebacterium glutamicum*, a
- 5 sufficient reporter for protein secretion.

#### Example 7

Mutation within the GSP diminish or abolish amylase secretion

- To quantify the effects of the mutations on protein export,
- 10 the strains *Corynebacterium glutamicum* INT-D and *Corynebacterium glutamicum* INT-F were transformed with the replicative plasmid pAmy. Starch degradation was tested on solid medium and in liquid culture. No secretion is detectable in *Corynebacterium glutamicum* INT-D/pAmy and
- 15 *Corynebacterium glutamicum* INT-F/pAmy. Determination of volume activity resembles the same phenotype no starch degradation could be found for both strains.

- Due to the surprising fact of a complete loss of the ability to secrete the heterologous amylase, the other sec genes of *Corynebacterium glutamicum* were mutated. Producing mutants of secA, secY and secE failed which are essential for cell viability, as pointed out above. To mutate secG, the wild type gene was replaced by a secG disrupted with the choramphenicol resistance cassette cmx via double
- 20 crossover using the sacB system. The resulting mutant strain *Corynebacterium glutamicum* INT-G (Table 1) grows normal at permissive temperatures but its cell wall is very sensitive against SDS (data not shown). All mutation were verified by Southern hybridisation.
- 25
- 30 Like the strains *Corynebacterium glutamicum* INT-D and *Corynebacterium glutamicum* INT-F, *Corynebacterium glutamicum* INT-G was transformed with the replicative plasmid pAmy to analyse the effect of the gene disruption

on the protein export. Starch degradation was tested on solid medium and in liquid culture. Amylase secretion is significantly diminished in *Corynebacterium glutamicum* INT-G/pAmy, compared with *Corynebacterium glutamicum*

- 5 RES167/pAmy: The mutant strain secretes only 21,5 % of the amylase exported by *Corynebacterium glutamicum* RES167/pAmy.

None of the strains showed any amylase activity in the cytoplasm. Due to this, we conclude that an intact secD and secF are inevitable for the export of the heterologous  
10 amylase. SecG is not essential for protein translocation itself but strongly influences the rate of export.

#### Example 8

Overexpression of combination of sec genes increases amylase secretion

- 15 Since SecD and SecF seems to be strong effectors on amylase secretion, the consequence of combined overexpression of secD and secF on protein export was examined. The combination secD and secF was cloned with SalI into the shuttle expression vector pEC-XK99A as described above.
- 20 *Corynebacterium glutamicum* AMY2 was transformed with the resulting plasmids pSecDF and tested for amylase activity. As pointed out in figure 3, the simultaneous overexpression of secD and secF genes enhanced the amylase secretion 1,5 fold in contrast to *Corynebacterium glutamicum* AMY2.
- 25 To analyze the effect of parallel overexpression of auxiliary sec genes secD and secF with essential sec genes, secE and secY EcoRI were cloned into pSecDF, resulting in the plasmids pSecEDF and pSecYDF as described in example 3. SecE and SecY were cloned, because their interaction with
- 30 the SecD/SecF complex is described in *E. coli* (Sagara, K., Matsujama, S. and Mitzushima, S., 1994, J. Bact. 176, 4111-4116). Test on amylase secretion revealed a 2,3 fold increase for *Corynebacterium glutamicum* AMY2/pSecEDF and



gained 2,5 fold for *Corynebacterium glutamicum* AMY2/pSecYDF compared with *Corynebacterium glutamicum* AMY2 (fig. 3).

Detection of amylase activity on solid medium indicates the same findings. After 24 h the starch degradation was more  
5 progressed for *Corynebacterium glutamicum* AMY2/pSecEDF and *Corynebacterium glutamicum* AMY2/pSecYDF have higher doubling rates than *Corynebacterium glutamicum* AMY2.

Growth in minimal medium with starch as sole carbon source mirrored these results: No growth is detectable for  
10 *Corynebacterium glutamicum* RES167 due to its inability to convert starch to a metabolizable form. *Corynebacterium glutamicum* AMY2/pSecEDF and *Corynebacterium glutamicum* AMY2/pSecYDF, which revealed the highest amylase secretion, showed the fastest growth of all tested strains and reached  
15 a slightly higher OD. This indicates that growth on starch is directly proportional to amylase secretion.